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***Streptococcus thermophilus* NCIMB 41856 ameliorates signs of colitis in an animal model of inflammatory bowel disease**

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**Running head:** *S. thermophilus* NCIMB 41856 ameliorates colitis

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## Abstract

**Background:** Treatment of inflammatory bowel disease (IBD) is mainly based on suppression of symptoms, often with numerous side effects. Trials of probiotics in IBD have frequently produced disappointing results. The majority of probiotics are unusual, since they do not require iron for growth, unlike many bacteria resident in the intestine. The IBD intestine is iron-rich due to bleeding and use of oral iron supplements; conventional probiotics would be rapidly outcompeted. We have evaluated an iron-responsive *Streptococcus thermophilus* strain for its potential to reduce signs of colitis.

**Methods:** Efficacy of *S. thermophilus* was evaluated in the dextran sodium sulphate (DSS) mouse model of colitis. Treated animals were given  $1 \times 10^8$  cfu *S. thermophilus* per day and clinical observations were taken daily. At termination, gross and histopathological signs of disease, cellular infiltration, location of bacteria, and cytokine expression in the intestine were determined.

**Results:** *S. thermophilus* delayed onset of colitis and reduced clinical signs of disease, including bodyweight loss and gastrointestinal bleeding. It reduced bacterial translocation into the colonic tissue. Increased numbers of CD8<sup>+</sup> intraepithelial lymphocytes were seen in control animals treated with *S. thermophilus*. *S. thermophilus* had no effect on gross pathology, histopathology or cytokine production in either colitic or control animals.

**Conclusions:** We propose that *S. thermophilus* promotes maintenance of mucosal barrier function which reduces bacterial translocation, thereby reducing immune stimulation and associated inflammation. This allows mucosal healing, reducing gastrointestinal bleeding and weight loss. This could be studied as a locally-acting adjunct or alternative to current IBD treatments.

**Key words:** Probiotic, epithelial barrier function, gastrointestinal bleeding.

## Introduction

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic, debilitating diseases affecting an estimated 2.5-3 million people in Europe (Burisch *et al.*, 2013). These diseases are characterised by relapsing and remitting inflammation and ulceration in the gastrointestinal tract. In many cases, these chronic inflammatory processes lead to irreversible intestinal damage, the only treatment for which is surgical resection. Current IBD treatments are mainly based on suppression of symptoms by the use of biologics, systemic anti-inflammatory drugs such as mesalazine, glucocorticoids or immunosuppressive agents; these therapies are associated with numerous side effects and therefore new treatment options with fewer side effects would be beneficial to patients.

The causes of IBD are not yet fully understood but one hypothesis is that a dysregulated mucosal immune response is initiated by an environmental factor in a genetically susceptible host (Kucharzik *et al.*, 2006; Xavier and Podolsky, 2007). It has been suggested that gut microbes, both pathogenic and commensal, could play a role in this. The intestinal microflora is fundamental in the maintenance of host health, but IBD patients have an altered intestinal microflora with decreased biodiversity (specifically reduced Bacteroidetes and Firmicutes, and increased numbers of *Escherichia coli* (Packey and Sartor, 2009)). In an attempt to redress this balance, reduce inflammation and the symptoms of IBD, probiotics, defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host', have been trialled. Trials have mainly focussed on *Lactobacillus* and *Bifidobacteria* spp. and, for Crohn's disease patients, have not produced the beneficial effects anticipated, with no consistent effects seen in treating active disease or preventing relapse of disease (Sanders *et al.*, 2013). Probiotics have shown more promise in the treatment of ulcerative colitis. Two preparations in particular, *E. coli* Nissle 1917 and VSL#3 (a combination of 8 different probiotic strains) have been shown to induce and maintain remission in mild to moderate disease (Kruis *et al.*, 2004; Miele *et al.*, 2009; Sood *et al.*, 2009). Despite the lack of clinical evidence for efficacy of many probiotic strains, in a recent study, 40% of IBD patients regularly used non-prescribed probiotics, and use was more common in Crohn's disease patients than ulcerative colitis patients (Agathou and Beales, 2013).

The lactic acid bacteria, frequently employed as probiotics, are unusual organisms in that they do not appear to have a requirement for iron (Bruyneel *et al.*, 1989; Pandey *et al.*, 1994; Imbert and Blondeau, 1998). This is in contrast to the majority of constituents of the commensal flora and many pathogens which are able to use iron to increase their growth rate (Wooldridge and Williams, 1993; Freestone *et al.*, 1999; Cogan *et al.*, 2007; Kortman *et al.*, 2012). Therefore, under high iron conditions, many lactic acid bacteria-based probiotics are rapidly outcompeted as other species increase growth rate and predominate. Inflammatory bowel disease sufferers have particularly iron-rich intestines due to intestinal bleeding, resulting from ulceration and chronic inflammation (Sturniolo *et al.*, 1998), and oral iron supplements taken to counteract anaemia. A recent European study found that 92% of anaemic IBD patients received iron supplementation; only 28% received intravenous iron whereas 67% had oral iron supplements (Stein *et al.*, 2013). We hypothesise that the increased levels of iron in the IBD gut are detrimental to conventional probiotics and could skew the microflora towards an unfavourable, potentially pathogenic population, which would provoke or maintain inflammation.

We previously identified an isolate of *Streptococcus thermophilus* (NCIMB 41856) with the ability to reduce epithelial cell death, maintain tight cell junctions, reduce pathogen binding to

epithelial cells, and reduce pro-inflammatory signalling from epithelial cells and intestinal leukocytes *in vitro* (Bailey *et al.*, 2011). This strain is unusual amongst lactic acid bacteria as it has the ability to increase its growth rate in response to iron (Bailey *et al.*, 2011). While iron is not required for *S. thermophilus* NCIMB 41856 to exert its beneficial effect, the ability to use it as a growth factor better equips it to compete with the resident flora and pathogens in the iron-rich environment of the IBD gut. We propose that this iron-responsive bacterium could be used therapeutically to reduce inflammation and promote mucosal healing in IBD patients. The aim of this study was therefore to evaluate the ability of *S. thermophilus* NCIMB 41856 to ameliorate colitis in an animal model of inflammatory bowel disease.

## Materials and Methods

### *Preparation of Streptococcus thermophilus NCIMB 41856*

*S. thermophilus* NCIMB 41856 was cultured in M17 broth supplemented with lactose (Oxoid, Cambridge, UK) at 37°C, under microaerobic conditions, overnight. Bacteria were washed with PBS and resuspended to a concentration of  $5 \times 10^8$  cfu/ml in 3% sodium bicarbonate. We have used the maximum dose possible in order to determine any positive effect in the DSS colitis model used. Bacteria were resuspended in 3% sodium bicarbonate in order to mimic the effect of administering encapsulated bacteria in humans.

### *Induction of colitis and treatment protocol*

All animal experiments were carried out by KWS BioTest Ltd (Bristol, UK) in accordance with UK Home Office Guidelines. Adult male BALB/c mice were housed under specific pathogen free conditions with food and water available *ad libitum*. Animals were randomised into four experimental groups of 10 mice according to bodyweight and allowed to acclimatise for one week. Starting from Day -2 and continuing until the end of the experiment (Day 11), two groups of animals were given a dose of  $1 \times 10^8$  cfu of *S. thermophilus* NCIMB 41856 in 3% sodium bicarbonate by oral gavage; two groups of control animals were given an equal volume of sodium bicarbonate alone. On Day 0, the drinking water was replaced with a 5% dextran sodium sulphate salt (DSS) solution for two groups, one given *S. thermophilus*, the other given sodium bicarbonate. Animals were given *ad libitum* access to the 5% DSS solution until the end of the experiment on Day 11. The experiment was carried out once with portions of the same colon being used for all analyses.

### *Clinical observations*

From Day 0 until the end of the experiment on Day 11, animals were monitored daily for clinical signs of colitis including bodyweight loss, loose stools and/or diarrhoea and presence of occult or gross blood in the stools. Animals were scored from 0-4 for weight loss (0 indicated no weight loss, 1 was a loss of 1-5%, 2 was 5-10%, 3 was 10-15% and 4 was greater than 15%), stool consistency (0 indicated normal stool, 2 was loose stool and 4 was diarrhoea) and bleeding (0 was no bleeding, 2 was hemocult and 4 was gross bleeding). Scores for each parameter were added together to give a total clinical score with a maximum score of 12. All data were assessed for normality and analysed by one-way MANOVA with an LSD post-hoc test (SPSS, IBM, Armonk, NY, USA).

### *Gross pathology*

At termination, colons were dissected out and a picture taken to allow for length measurements to be performed on digital images using ImageJ software

(<http://rsb.info.nih.gov/ij>). Data were assessed for normality and analysed by one-way ANOVA with a Tukey post-hoc test (GraphPad Prism 5, California, USA).

#### *Histopathology*

Colons were dissected out and a small pieces of tissue was stored at ambient temperature in tissue fixative, without prior washing, for histopathology analysis. Samples were processed for paraffin embedding and sections of colon were cut and stained with haematoxylin and eosin (H&E). Sections were scored on a scale of 0 to 2 by a qualified histopathologist for signs of colitis, specifically mucosal thickness, mucosal ulceration, lamina propria mononuclear cell infiltration, granulocyte infiltration and crypt abscesses and/or dilation and/or distortion. The scoring system was based on assessment of overall sections and the maximum possible score was 10. Data were assessed for normality and analysed by one-way ANOVA with a Tukey post-hoc test (GraphPad Prism 5).

#### *Fluorescence in situ hybridisation to quantify and identify location of *S. thermophilus* and total bacteria in colon tissue*

Fluorescence in situ hybridisation (FISH) to *S. thermophilus* was carried out as previously described (Garcia-Hernandez *et al.*, 2012) on sections of formalin-fixed colon using a Cy3-conjugated probe (5'-CATGCCCTTCGCTTACGCT-3') specific to 23S rRNA. Total levels of bacteria were quantified using a combination of three FITC-conjugated eubacteria probes to 16S rRNA (EUB338 5'GCT GCC TCC CGT AGG AGT-3', EUB 338 II 5'GCA GCC ACC CGT AGG TGT-3, and EUB338 III 5'GCT GCC ACC CGT AGG TGT-3') as previously described (Daims *et al.*, 1999). Bacteria adherent to the epithelium and within the tissue were counted. Data were not normally distributed and were therefore analysed by Kruskal-Wallis test with a Dunn's multiple comparison post-hoc test (GraphPad Prism 5).

#### *Immunofluorescence to determine level of expression of CD8 in colon*

Colons were dissected out and a small piece of tissue was mounted in OCT and snap frozen in liquid nitrogen without prior washing. 5µm sections were fixed in acetone and blocked with IHC/ICC blocking buffer (eBioscience, San Diego, California, USA). Sections were incubated overnight at 4°C with rat anti-mouse CD8α (1/200; eBioscience). FITC-conjugated anti-rat IgG (1/200; eBioscience) was used to detect CD8α and sections were incubated for 90 minutes at room temperature. Sections were mounted in Vectashield containing DAPI (Vector Laboratories, California, USA) and visualised on a Leica DMRA microscope equipped with a Hamamatsu Orca-ER monochrome camera. Leica Q-Fluoro software was used to capture ten fields of view per section at 40x magnification. Images were viewed using ImageJ software (<http://rsb.info.nih.gov/ij>) and positive pixels automatically counted as previously described (Inman *et al.*, 2005). Data were assessed for normality and analysed by one-way ANOVA with a Tukey post-hoc test.

#### *RT-qPCR to determine IL-6 and IL-17a expression*

RNA was extracted from colon samples stored in RNeasy later using an RNeasy mini kit (Qiagen, Crawley, UK). Synthesis of cDNA was carried out using 500ng random hexamers and the ImProm-II Reverse Transcription System (Promega, Southampton, UK) in a final volume of 20µl. All reactions were prepared according to the manufacturer's instructions giving a final magnesium concentration of 3mM. All cDNAs were diluted to a final volume of 100µl (1/5 dilution) using EB buffer (10mM Tris HCl pH 8.4; Qiagen). Primers and probes were designed using Primer3 (<http://frodo.wi.mit.edu/primer3>) and M-fold

(<http://mfold.rna.albany.edu/?q=mfold>) using the mouse specific GenBank sequences for IL-6 (accession number NM\_031168) and IL-17a (accession number NM\_010552). The housekeeper gene GAPDH was used as an internal control. Primer and probe sequences are shown in Table 1. Quantitative PCR (qPCR) was performed using GoTaq master mix (Promega). Gene specific amplification was performed using 0.2µM of each primer, 0.1µM of probe and 5µl of diluted cDNA in a final volume of 25µl. Magnesium chloride concentrations were adjusted to 4.5mM in the final reaction by addition of 50mM MgCl<sub>2</sub>. Amplifications were performed in an MxPro3005P (Stratagene, California, USA) at 95°C for 2 minutes and then 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds during which time the fluorescence data was collected. Data were assessed for normality and analysed by one-way ANOVA with a Tukey post-hoc test (GraphPad Prism 5).

**Table 1:** Primers used to quantify transcription of IL-6 and IL-17a

Name	Primer and probe sequences	
IL-6	Forward	5'-AGCCAGAGTCCTTCAGAGAGA-3'
	Reverse	5'-ACTCCTTCTGTGACTCCAGC-3'
	Probe	5' FAM-CCCCAATTTCCAATGCTCTCC-3' BHQ1
IL-17a	Forward	5'-CCAAACACTGAGGCCAAGGA-3'
	Reverse	5'-TGAGGTAGTCTGAGGGCCTT-3'
	Probe	5' FAM-GTCTTTAACTCCCTTGGCGC-3' BHQ1
GAPDH	Forward	5'-GTCATCCCAGAGCTGAACGG-3'
	Reverse	5'-GCCTGCTTCACCACCTTCTT-3'
	Probe	5' FAM-TTCCTACCCCCAATGTGTCC-3' BHQ1

## Results

### *S. thermophilus* reduces clinical signs of colitis

Figure 1: DSS induced signs of colitis including loss of bodyweight (A), increase in clinical score (B), increase in faecal blood score (C) and an increase in faecal consistency score (D).

Animals were weighed daily to monitor DSS-induced loss of bodyweight. DSS administration induced a highly significant bodyweight loss from Day 3 until the end of the experiment on Day 11 when the total weight loss was 13.5% ( $\pm 6.3$ ) compared to initial bodyweight. While colitic animals given *S. thermophilus* did still lose a significant percentage of bodyweight, the onset of this was delayed until Day 9 and the final weight loss was significantly lower at 7.2% ( $\pm 8.4$ ); *S. thermophilus* administration significantly reduced DSS-induced bodyweight loss at Day 4 and Days 7 to 11 (Figure 1A). *S. thermophilus* administered to healthy animals did not induce any weight changes.

Clinical signs of colitis (combination of bodyweight loss, loose stools and/or diarrhoea and presence of occult or gross blood in the stools) were monitored daily and scored on a scale of 0-12 where 0 indicates a healthy animal and 12 indicates severe colitis. DSS-administered animals first started to show disease signs on Day 2; by Day 8 clinical scores were significantly higher in DSS-treated animals than controls. Animals treated with DSS and *S. thermophilus* developed colitis later, at Day 9, and the clinical scores were significantly lower than those of DSS-treated animals at Day 8 and Day 10 (Figure 1B). The administration of *S. thermophilus* to healthy animals did not induce any increase in clinical score.

Animals dosed with DSS showed presence of blood in faeces, indicating significant gastrointestinal bleeding, on Day 8 and this continued until the end of the experiment on Day 11. DSS-treated animals given *S. thermophilus* did not show observable blood in faeces until Day 10 and, at this point, the amount of blood was significantly lower than that in animals given DSS alone (Figure 1C). DSS induced significant levels of loose stools on Day 9 and this progressed towards diarrhoea as the experiment continued. *S. thermophilus* treatment had no effect on faecal consistency of DSS-treated animals. Control animals given *S. thermophilus* showed signs of mild loose stools on Days 3, 4 and 10 (Figure 1D).

#### *The effect of DSS and S. thermophilus administration on pathological markers of inflammatory disease*

Figure 2: Treatment with *S. thermophilus* had no effect on DSS-induced colon shortening (A) or histopathology score (B).

Colon shortening is associated with severity of colitis and the administration of DSS induced a shortening of the colon when compared to the control group which was not prevented by *S. thermophilus* treatment. *S. thermophilus* treatment alone did not induce any reduction in colon length (Figure 2A). The nature of pathological change was as expected for the DSS model. The most severely affected colons displayed mucosal thickening, full circumferential ulceration, marked mixed mononuclear and granulocytic infiltration with inflammation and oedema of the submucosa, with loss or distortion of cryptal structure. DSS induced a highly significant increase in histopathology scores but administration of *S. thermophilus* was unable to prevent this. *S. thermophilus* treatment of healthy animals did not induce any histopathological changes (Figure 2B).

#### *S. thermophilus reduces binding of bacteria to epithelial cells and bacterial translocation*

Figure 3: Adherence of *S. thermophilus* (A) and non-*S. thermophilus* bacteria (B) to colonic epithelium.

*S. thermophilus* was found bound to the colonic epithelium of both DSS-treated and control mice (Figure 3A). In healthy animals, *S. thermophilus* treatment significantly reduced levels of non-*S. thermophilus* bacteria bound to the epithelium but it did not significantly reduce the number of non-*S. thermophilus* bacteria bound to the epithelial cells in colitic animals (Figure 3B). Although not significant, we tended to see lower levels of bacteria associated with the epithelium in DSS-treated animals compared to controls. We hypothesise that this is due to a higher degree of epithelial cell shedding and therefore a reduction in the availability of bacterial binding sites. In 40% of DSS-treated animals and 30% of control animals, *S. thermophilus* was able to cross the epithelial barrier and penetrate into the colonic tissue (Figure 4A). In DSS-treated animals, *S. thermophilus* treatment significantly reduced translocation of non-*S. thermophilus* bacteria into the colonic tissue (Figure 4B). *S. thermophilus* was not found at any location in animals which had not received *S. thermophilus* NCIMB 41856 (data not shown).

Figure 4: Translocation of *S. thermophilus* (A) and non-*S. thermophilus* bacteria (B) into the colonic tissue.

#### *The role of S. thermophilus in the mucosal immune response*

Figure 5: DSS induced an upregulation of IL-6 (A) and IL-17 (B) transcripts in the colon.



DSS induced an upregulation of both IL-6 and IL-17 transcripts in the colon, as expected for this model (Alex *et al.*, 2009). The administration of *S. thermophilus* was unable to prevent this but there was no effect on the production of these cytokines in healthy animals given *S. thermophilus* (Figure 5). In healthy animals, *S. thermophilus* treatment was associated with increased expression of CD8 (Figure 6A) and these cells were predominantly resident within the epithelium, indicating that they are likely to be intraepithelial lymphocytes (Figure 6B).

Figure 6: *S. thermophilus* treatment increased expression of CD8 in control animals (A), predominantly in the epithelium.

## Discussion

The predominant feature of IBD is inflammation in the gastrointestinal tract, either confined to the colon in ulcerative colitis or at any point from mouth to anus in Crohn's disease. This inflammation manifests itself as recurrent or bloody diarrhoea, abdominal pain and weight loss. Using an animal model of disease we have assessed the efficacy of *S. thermophilus* NCIMB 41856 to reduce inflammation and alleviate IBD signs. While *S. thermophilus* treatment had no effect on faecal consistency, it did significantly reduce weight loss and presence of blood in faeces (Figure 1), two common symptoms of IBD.

We conducted a pilot experiment to evaluate the ability of both the iron responsive *S. thermophilus* strain NCIMB 41856 and a non-iron responsive *S. thermophilus* strain to survive and persist within the mouse intestine. Both strains were given in equal doses in 3% sodium bicarbonate by oral gavage to groups of three healthy mice. Three days post gavage, *S. thermophilus* NCIMB 41856 could be found in 2/3 mice whereas the non-iron responsive strain was not detected in any animal (unpublished data). While not significant, we felt this gave us a strong enough indication that a non-iron responsive *S. thermophilus* was not able to survive in the mouse intestine and therefore we did not investigate it further. In contrast, *S. thermophilus* NCIMB 41856 was able to adhere to the colonic epithelium in both control and DSS-treated animals and binding of other bacterial species to the epithelium was reduced (Figure 3). Competitive exclusion of pathogens from epithelial surfaces has been proposed as a mechanism by which probiotics may act. Competition both for limited nutrients and physical attachment sites could assist in the elimination of pathogens and opportunistic pathogens. We have previously shown that, unlike the majority of conventional probiotics, *S. thermophilus* NCIMB 41856 can compete with the resident microflora for iron, abundant in the IBD gut and used by many bacterial strains as a growth factor (Bailey *et al.*, 2011). In the data presented here, we have shown that, in addition to this, it can compete for physical attachment sites at the iron-rich intestinal mucosa and therefore occupy a niche which could otherwise be inhabited by potentially pathogenic bacterial species.

Epithelial cells lining the gastrointestinal tract form the first line of defence against invading pathogens. Epithelial barrier function is known to be disrupted in IBD patients (Irvine and Marshall, 2000; Pastorelli *et al.*, 2013) and those with a higher degree of intestinal permeability have been shown to be at a greater risk of relapse (Wyatt *et al.*, 1993; Arnott *et al.*, 2000; Shavrov *et al.*, 2015). Defective barrier function exacerbates diarrhoea and allows for increased uptake of luminal bacteria which results in a loss of tolerance to the resident microflora, thus stimulating the underlying immune system, enhancing and maintaining chronic intestinal inflammation (Duchmann *et al.*, 1995). Tight junctions, found between epithelial cells, are required to maintain adequate barrier function but defects in the expression of tight cell junction proteins have been observed in IBD patients. Decreases in expression of the claudins, ZO-1, occludin and  $\alpha$ -catenin have been seen in both Crohn's disease and ulcerative colitis patients (Heller *et al.*, 2005; Zeissig *et al.*, 2007; Vivinus-Nebot

*et al.*, 2014). Additionally, increased leaks which occur as a result of epithelial cell apoptosis have been observed in Crohn's disease and ulcerative colitis (Heller *et al.*, 2005; Zeissig *et al.*, 2007). We have previously shown that *S. thermophilus* prevents occludin degradation and breakdown of tight cell junctions induced by *E. coli* *in vitro* as well as reducing epithelial cell death (Bailey *et al.*, 2011). In the data presented here, we have shown that *S. thermophilus* prevents bacterial translocation from the lumen of the gut into the intestinal tissue in colitic animals (Figure 4). We hypothesise that this is due to the ability of *S. thermophilus* to protect and maintain tight cell junctions and prevent epithelial cell apoptosis, thereby enhancing barrier function.

In addition to the direct effects of *S. thermophilus* on maintenance of tight cell junctions and barrier function, healthy animals treated with *S. thermophilus* showed increased numbers of CD8<sup>+</sup> intraepithelial lymphocytes (IELs; Figure 6). The picture in colitic animals was less clear since DSS induces a generalised increase in intestinal CD8<sup>+</sup> cells (Sund *et al.*, 2005). IELs reside within the intestinal epithelial barrier and are predominantly CD8<sup>+</sup>. These cells play a vital role in host defence at the mucosal surface and facilitate maintenance of a competent intestinal barrier. For example, they have been shown to modulate expression of tight cell junction proteins by epithelial cells in order to maintain barrier integrity following infection (Dalton *et al.*, 2006; Inagaki-Ohara *et al.*, 2006). Furthermore, they regulate proliferation and differentiation of epithelial cells and play a role in induction of apoptosis in senescent or infected cells, allowing for rapid regeneration and reducing the risk of leaks (Guy-Grand *et al.*, 1998). IELs are a mixed population of cells with different phenotypes having different roles in intestinal inflammation, some of which have been shown to be protective against colitis in animal models of disease (Chen *et al.*, 2002; Inagaki-Ohara *et al.*, 2004; Roselli *et al.*, 2009). Further phenotypical and functional analyses are required in order to accurately determine the role that this increased population of CD8<sup>+</sup> IELs play in inflammation. However, given the reduction in bacterial translocation seen in this colitis model following *S. thermophilus* treatment, it seems likely that these IELs are of a protective rather than a colitogenic phenotype. Since we have previously shown that *S. thermophilus* is able to maintain tight cell junctions *in vitro* in the absence of IELs (Bailey *et al.*, 2011), we hypothesise that it exerts two different mechanisms to enhance mucosal barrier integrity: preservation of occludin expression and increasing numbers of IELs which are multifunctional in maintaining a competent epithelial barrier.

DSS induced an upregulation in transcription of IL-6 and IL-17 and histopathological signs of inflammation but *S. thermophilus* had no effect on this (Figure 5). DSS is a polyclonal activator of T and B cells (Ni *et al.*, 1996) therefore it is not surprising that *S. thermophilus* is unable to prevent this direct activation, subsequent cytokine production and inflammation from occurring. We hypothesise that a stronger epithelial barrier, enhanced by *S. thermophilus*, reduces bacterial translocation, thereby reducing antigen-specific immune cell activation and secondary inflammation but it is unable to prevent the direct activation of immune cells by DSS.

We have shown that *S. thermophilus* treatment of DSS-induced colitis in mice had a clear beneficial effect. DSS-induced colitis is frequently used as a model of human IBD due to its simplicity and many similarities with the natural disease. However, it is not a perfect model and the mechanism by which it induces inflammation is not entirely understood. It is thought that inflammation results from damage to the epithelial cells lining the colon which allows dissemination of pro-inflammatory intestinal contents into the tissue (Chassaing *et al.*, 2014). In this study, animals were being treated with DSS for the duration of the experiment; it was a progressive model with a continuous chemical assault on the epithelial cells lining the gut,

therefore, a cure of colitis by *S. thermophilus* would not be expected. We have used this model to look for markers of efficacy rather than total resolution of inflammation and clinical signs. We would like to evaluate the efficacy of *S. thermophilus* NCIMB 41856 to reduce signs of colitis in a spontaneous mouse model of colitis, such as the SAMP1/YitFc mouse (Matsumoto *et al.*, 1998; Sugawara *et al.*, 2005) or the recently developed Winnie mouse (Heazlewood *et al.*, 2008). This will allow us to further investigate parameters such as the cytokine inflammatory profile in models more comparable to the naturally-occurring disease.

The goal of IBD treatment is to reduce inflammation early in the course of disease and induce mucosal healing. Here we have shown that treatment with *S. thermophilus* NCIMB 41856 was able to reduce two key signs of IBD in an animal model of disease: weight loss and intestinal bleeding. We suggest that these key physical effects are mediated by enhanced epithelial barrier function, demonstrated by reduced bacterial translocation, induced by *S. thermophilus*. The mucosal barrier is often disrupted in IBD patients leading to increased intestinal permeability; therefore a more robust barrier would significantly lessen bacterial translocation and prevent antigenic overload, thereby reducing inflammation. While other probiotics have been shown to enhance barrier function (Madsen *et al.*, 2001; Luyer *et al.*, 2005; Zeng *et al.*, 2008; Pagnini *et al.*, 2010; Zakostelska *et al.*, 2011; Carlsson *et al.*, 2013), they are unlikely to be able to survive and compete within the iron-rich environment of the IBD intestine. Since *S. thermophilus* NCIMB 41856 can use iron as a growth factor it is able to compete with the resident microflora in order to survive and exert its beneficial effect (Bailey *et al.*, 2011).

Conventional IBD treatments are frequently immunosuppressive and often act systemically, leaving the patient more susceptible to infection. We propose that *S. thermophilus* NCIMB 41856 could show utility as an alternative to current IBD treatments or as an adjunct which may improve the efficacy of conventional drugs, allowing the same level of control with a lower dose of systemic immunosuppressive therapy, thereby potentially reducing side effects. *S. thermophilus* NCIMB 41856 is not immunosuppressive, has shown no deleterious effects in healthy or colitic animals and would act locally to restore barrier function, preventing perpetual immune stimulation and therefore reducing inflammation. This, in turn, would reduce gastrointestinal bleeding and weight loss.

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## Figure legends

Figure 1: DSS induced signs of colitis including loss of bodyweight (A), increase in clinical score (B), increase in faecal blood score (C) and an increase in faecal consistency score (D). Treatment with *S. thermophilus* NCIMB 41856 delayed onset of colitis symptoms and significantly reduced loss of bodyweight, clinical score and faecal blood score but had no effect on faecal consistency. Data for each group are expressed as means. \* indicates  $p<0.05$ , \*\*  $p<0.01$ , \*\*\* $p<0.001$  when the DSS group is compared to control. # indicates  $p<0.05$ , ##  $p<0.01$  and ###  $p<0.001$  when the DSS group is compared to the DSS + *S. thermophilus* group.

Figure 2: Treatment with *S. thermophilus* had no effect on DSS-induced colon shortening (A) or histopathology score (B) but did not induce any pathological or histopathological changes alone. \* indicates  $p<0.05$  and \*\* $p<0.01$ .

Figure 3: Adherence of *S. thermophilus* (A) and non-*S. thermophilus* bacteria (B) to colonic epithelium. *S. thermophilus* adhered to the epithelium and binding of other species in control animals was reduced. \*\* indicates  $p<0.01$ .

Figure 4: Translocation of *S. thermophilus* (A) and non-*S.thermophilus* bacteria (B) into the colonic tissue. *S. thermophilus* treatment was associated with significantly reduced translocation of other bacterial species in colitic animals. \* indicates  $p<0.05$ .

Figure 5: DSS induced an upregulation of IL-6 (A) and IL-17 (B) transcripts in the colon. This was not affected by treatment with *S. thermophilus*; *S. thermophilus* had no effect on the transcription of either cytokine in control animals. \*\* indicates  $p<0.01$  and \*\*\* indicates  $p<0.001$ .

Figure 6: *S. thermophilus* treatment increased expression of CD8 in control animals (A), predominantly in the epithelium (CD8<sup>+</sup> cells are shown in green and nuclei are stained with DAPI) (B).



Figure 1

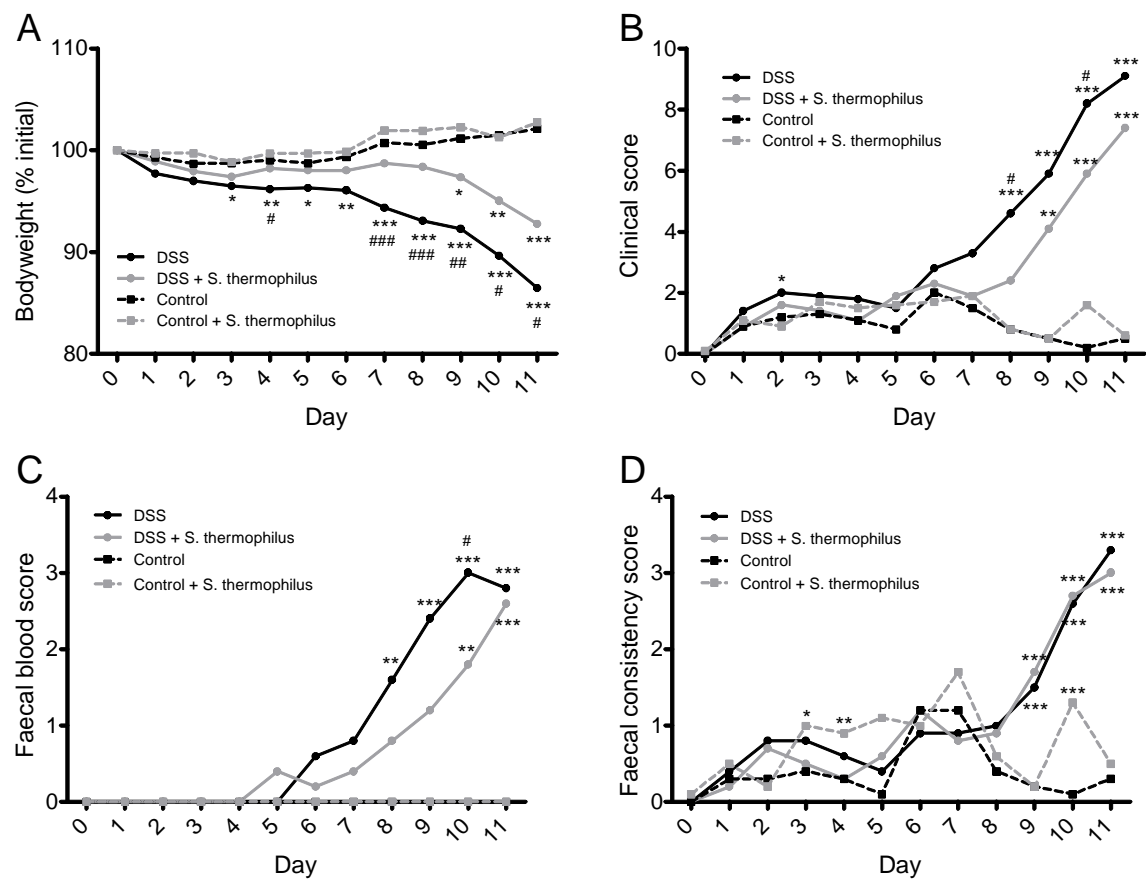


Figure 2

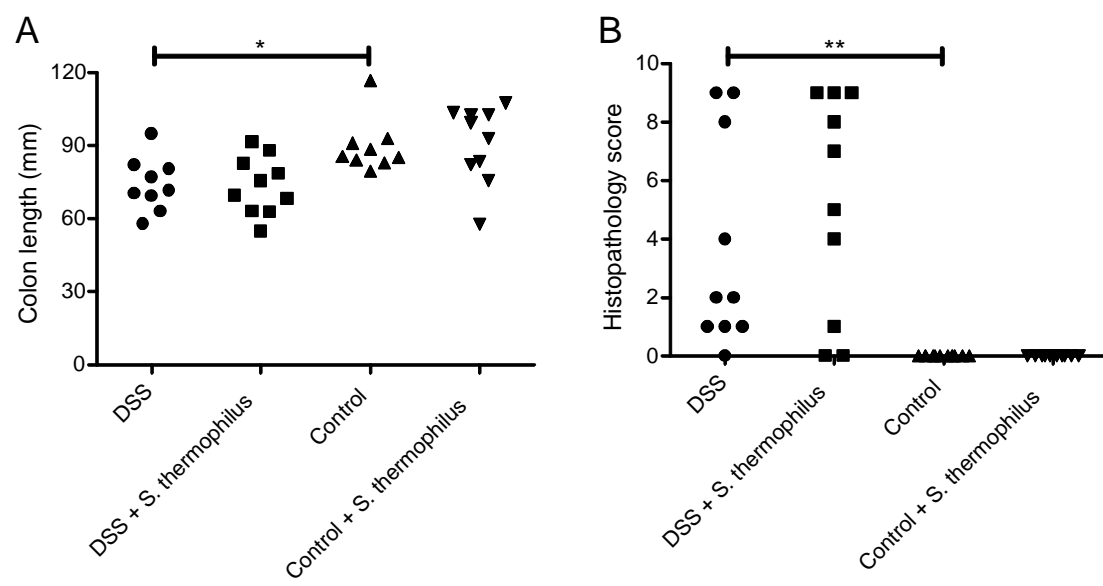


Figure 3

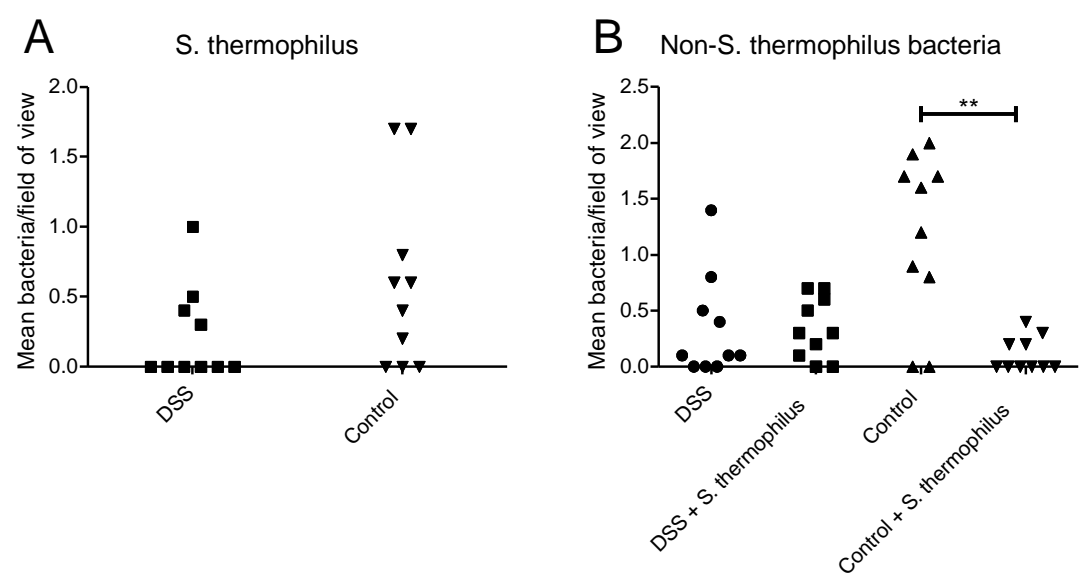


Figure 4

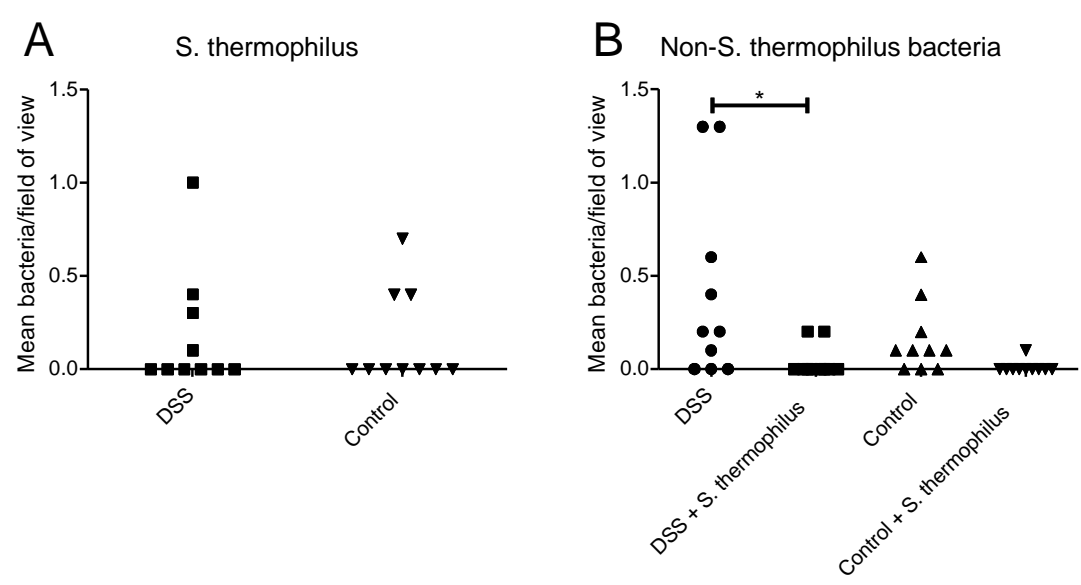


Figure 5

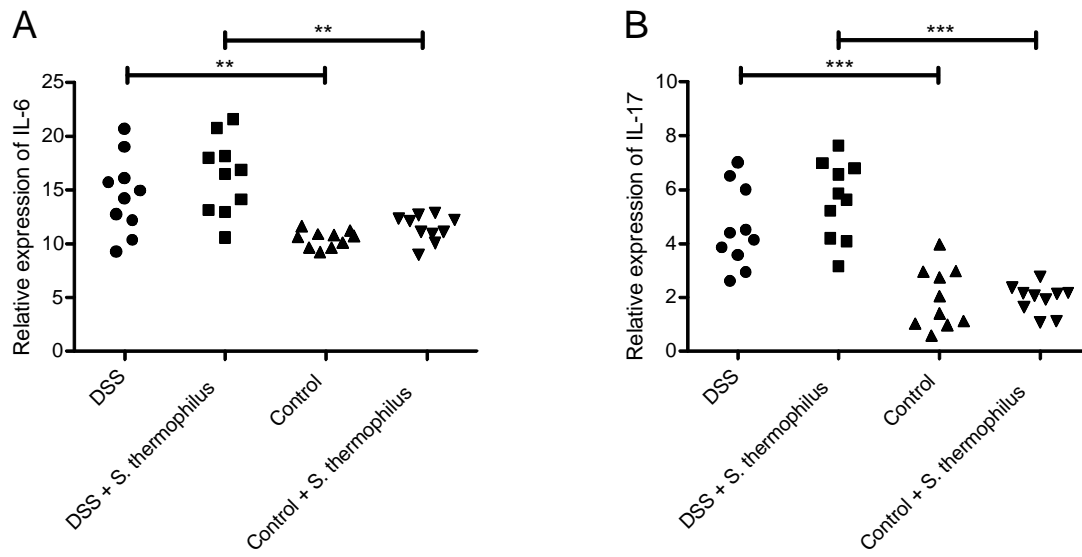


Figure 6

